The Plant Cell, Vol. 5, 603–613, June 1993 © 1993 American Society of Plant Physiologists

RESEARCH ARTICLE

Scaffold Attachment Regions Increase Reporter Gene Expression in Stably Transformed Plant Cells

George C. Allen,[a] Gerald E. Hall, Jr.,[b] Lisa C. Childs,[b] Arthur K. Weissinger,[c] Steven Spiker,[b] and William F. Thompson[a,b,1]

[a] Department of Botany, North Carolina State University, Raleigh, North Carolina 27695
[b] Department of Genetics, North Carolina State University, Raleigh, North Carolina 27695
[c] Department of Crop Science, North Carolina State University, Raleigh, North Carolina 27695

The yeast ARS-1 element contains a scaffold attachment region (SAR) that we have previously shown can bind to plant nuclear scaffolds in vitro. To test effects on expression, constructs in which a chimeric β-glucuronidase (*GUS*) gene was flanked by this element were delivered into tobacco suspension cells by microprojectile bombardment. In stably transformed cell lines, GUS activity averaged 12-fold higher (24-fold on a gene copy basis) for a construct containing two flanking SARs than for a control construct lacking SARs. Expression levels were not proportional to gene copy number, as would have been predicted if the element simply reduced position effect variation. Instead, the element appeared to reduce an inhibitory effect on expression in certain transformants containing multiple gene copies. The effect on expression appears to require chromosomal integration, because SAR constructs were only twofold more active than the controls in transient assays.

INTRODUCTION

Most genes whose expression has been studied in transgenic plants are expressed in generally appropriate patterns with respect to cell and organ specificity, developmental timing, and response to environmental cues (Weissing et al., 1988; Benfey and Chua, 1989). However, the level of expression can vary over an extremely wide range (Herrera-Estrella et al., 1984; An, 1985; Dean et al., 1988; Hobbs et al., 1990). Peach and Velten (1991) have recently reviewed data indicating that a majority of detectable transformation events result in very low expression and have argued that it is therefore reasonable to suppose that many more cases of successful DNA transfer go undetected.

Variation in transgene expression is frequently attributed to corresponding variation in the transcriptional potential of different chromosomal insertion sites. In at least one case (Al-Shawi et al., 1990), it was possible to reclone an introduced gene showing aberrant expression and to establish that it produced the full spectrum of expression levels in a secondary transformation assay. Thus, in this case, and presumably in many others, most of the original variation must be related to characteristics of the genomic insertion site.

Prominent among the many factors that may affect expression at different genomic sites is the higher order structure of chromatin. For example, the transcriptional potential of large regions is thought to be controlled by the extent to which the 30-nm chromatin fiber is unwound to the level of the 11-nm nucleosome fiber, thus permitting access to the DNA by RNA polymerase and transcriptional regulatory proteins. According to current models of eukaryotic chromatin structure, unwinding may be regulated independently in each of many different chromosomal domains (Goldman, 1988; Cook, 1989; Manuelidis, 1990; Jackson, 1991; Pienta et al., 1991). These domains are thought to correspond both to the domains classically defined by nuclease sensitivity mapping (Weisbrod, 1982; Reeves, 1984) and to "loop domains" visualized in electron microscope studies of partially deproteinized metaphase chromosomes and interphase nuclei (Paulson and Laemmli, 1977).

The electron micrograph studies also revealed a proteinaceous matrix or "scaffold" to which DNA is attached at intervals to form a series of loops, varying in sizes from ~10 to several hundred kilobases. A variety of observations supports the view that the scaffold attachment points function as domain boundaries and play an important role in regulating gene expression. Of particular interest are observations in animal systems that suggest scaffold attachment can insulate transgenes from the influence of surrounding chromatin. Certain DNA sequences called scaffold attachment regions (SARs) that bind to the nuclear scaffold in vitro have also been shown to reduce position effect variation in vivo when included on both sides of globin (Grosveld et al., 1987) or lysozyme (Stief et al., 1989; Bonifer

[1] To whom correspondence should be addressed at the Department of Botany.

et al., 1990) gene constructs. These and other data (reviewed in Elgin, 1991; Jackson, 1991; Pienta et al., 1991) have led to the idea that SARs define independent domains in which higher order chromatin structure is determined independently from that of the surrounding chromatin. In addition, SARs and perhaps other types of sequences (Kellum and Schedl, 1992) may act to reduce the influence of enhancers and other *cis*-acting regulatory elements near a given integration site.

Results generally compatible with this notion have recently been reported for a plant system by Breyne et al. (1992). These authors showed that a soybean DNA fragment with SAR activity could influence the expression of a chimeric β-glucuronidase (*GUS*) gene introduced into tobacco cells with an Agrobacterium T-DNA vector. The coefficient of variation for GUS enzyme activity was reduced approximately twofold in their sample of independently transformed callus lines. However, the plant SAR also reduced the average *GUS* expression by approximately the same amount, largely by eliminating the strongest expressers from the population. This result differs from reports in the animal literature, where reduced variability is generally accompanied by increased gene expression. Although the reason for this difference remains unclear, it might simply reflect the fact that T-DNA inserts are frequently found near expressed genes (Herman et al., 1990; Kertbundit et al., 1991) and thus in regions that can be presumed to already have a chromatin conformation favoring transcription. In such regions, SARs might reduce the influence of nearby positive *cis*-regulatory elements and thus decrease average expression levels relative to controls.

We report here a series of experiments designed to test the effect of a sequence with known SAR activity in a transformation system that does not rely on Agrobacterium vectors. We also sought to minimize possible effects of physical linkage between assayable and selectable markers by using a cotransformation protocol in which a reporter gene and selectable marker were introduced on separate plasmids. SAR activity was provided by the yeast ARS-1 element (Stinchcomb et al., 1979), which has been shown to bind specifically to nuclear scaffold preparations from yeast (Amati and Gasser, 1988; Amati et al., 1990) and tobacco (Hall et al., 1991). Our results showed that this element can increase average gene expression by more than 20-fold in stably transformed tobacco cell lines. Chromosomal integration appears to be required, because we observed only a small effect in transient expression assays. Expression levels in individual cell lines were not proportional to the number of integrated gene copies. Instead, we observed a complex relationship in which expression is maximal in a relatively narrow range of gene copy numbers.

RESULTS

Reporter Gene Expression

To avoid possible effects of Agrobacterium sequences and minimize physical linkage between assayable and selectable markers, we used a cotransformation protocol in which unlinked reporter gene and selectable marker plasmids were delivered by particle bombardment. Figure 1 shows the constructs used. The control plasmid contains a chimeric gene consisting of the cauliflower mosaic virus (CaMV) 35S promoter driving the *GUS* reporter with a nopaline synthase (*nos*) polyadenylation signal (35S::*GUS*::*nos*). Test constructs have SARs located either 5′, 3′, or both 5′ and 3′ (double SAR) of 35S::*GUS*::*nos*. The SAR is the yeast ARS-1 fragment. This fragment is known to bind specifically to the yeast nuclear scaffold (Amati and Gasser, 1988), and we have previously shown that it also binds specifically to nuclear scaffold preparations from the tobacco cell line used in our experiments (Hall et al., 1991).

Transformation was achieved by mixing appropriate plasmids, coprecipitating them onto microprojectiles, and bombarding plates of cells using a protocol similar to that described by Klein et al. (1988) and Russell et al. (1992). Kanamycin-resistant (Km^r) callus lines were selected as described in Methods, and pieces of each resistant callus mass were assayed for GUS activity by incubation in a solution of X-glucuronide (X-gluc).

Figure 2 shows the first 14 calli obtained for each construct in one such experiment, assayed by the histochemical procedure ~6 weeks after transformation. A total of 40 to 80 independent transformants per construct were ultimately analyzed in this experiment, with results similar to those shown in Figure 2. When cotransformation was performed with the control construct, less than 60% of the Km^r lines examined expressed *GUS* strongly enough to be detected in this assay. From evidence presented below, we believe that virtually all

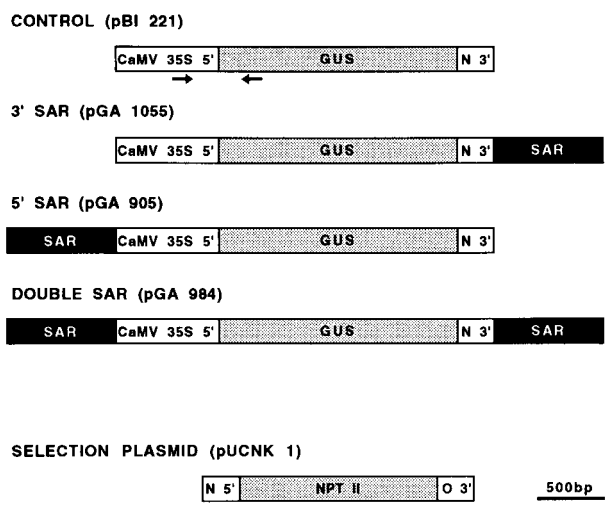

Figure 1. Schematic Diagrams of Plasmid Constructs.

CaMV 35S, cauliflower mosaic virus 35S promoter; *GUS*, coding region of the *E. coli* β-glucuronidase gene; N 3′, polyadenylation site/terminator from the nopaline synthase (*nos*) gene; SAR, scaffold attachment region (yeast ARS-1 element); N 5′, promoter from the *nos* gene; NPTII, *nptII* gene from Tn*5*; O 3′, polyadenylation site/terminator from the octopine synthase gene. The arrows indicate the locations of the PCR primers used in copy number estimation.

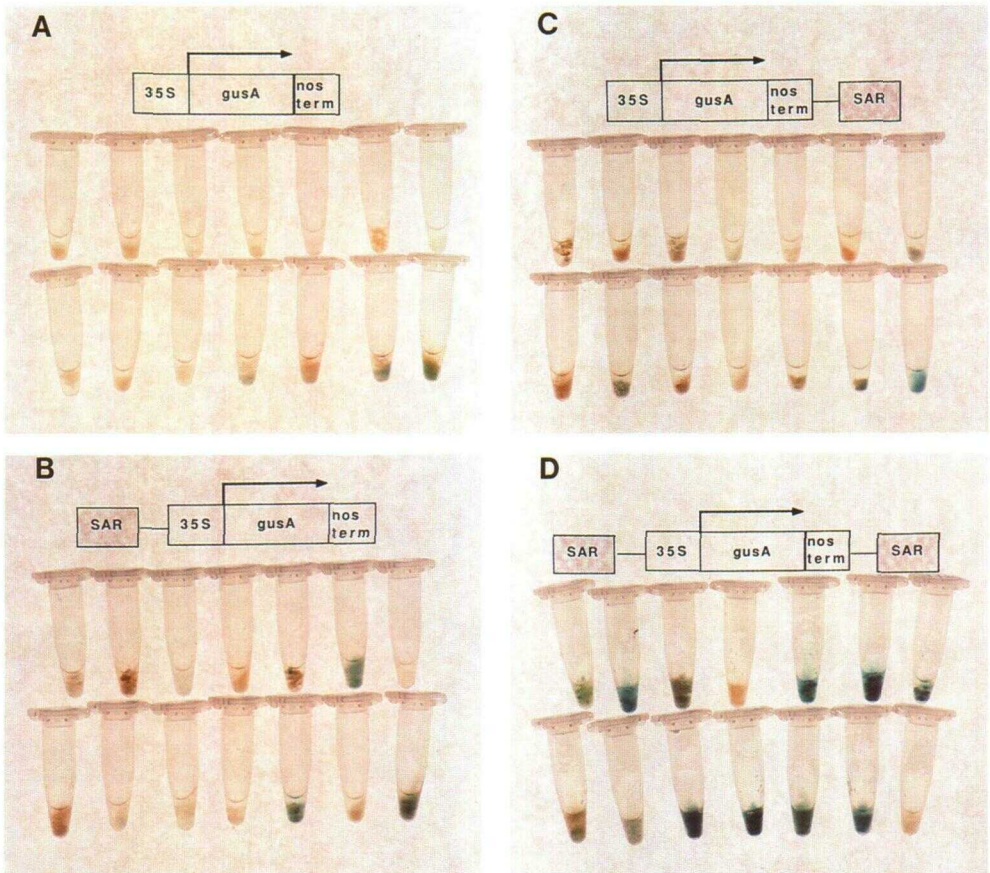

Figure 2. Flanking SARs Increase *GUS* Expression.

Plasmids containing derivatives of the 35S::*GUS* reporter gene with or without SARs together with a separate plasmid containing a kanamycin resistance gene were introduced into NT-1 tobacco suspension cells by microprojectile bombardment. The reporter plasmid was present in a 4:1 molar ratio to the selection plasmid to ensure that a high percentage of the resistant colonies would carry the unselected marker. Equimolar amounts of each of the different *GUS* plasmids were used in each transformation; in the case of the double SAR plasmid, the mixture contained a total of 1 μg of DNA per bombardment. Samples of calli from the first 14 Km^r cell lines generated with each construct are shown after staining for GUS activity with X-gluc. *nos term.*, *nos terminator.*

(A) Control reporter plasmid.

(B) 5′ SAR reporter plasmid.

(C) 3′ SAR reporter plasmid.

(D) Double SAR reporter plasmid.

of the cell lines expressing kanamycin resistance also contain *GUS* genes. However, in the case of the control construct, many of these lines failed to produce high enough levels of GUS to be detectable in this relatively insensitive assay. Constructs containing one or more SARs in addition to the 35S::*GUS reporter gene exhibited detectable GUS* expression in a greater percentage of the transformants. This effect is most dramatic in the case of the double SAR construct, in which 90% of the 41 Km^r calli tested were scored as positive for *GUS* expression and the levels of expression were often much higher than for the other constructs; 5′ or 3′ SAR constructs gave values of 75 to 80%. As shown in Figure 2, the average expression levels for these constructs were also somewhat lower than those obtained with double SAR constructs. Thus, we concluded that the presence of SARs in the *GUS* reporter construct can dramatically increase *GUS* expression and increase the percentage of transformants expressing *GUS* at detectable levels.

To provide a more quantitative analysis of the SAR effect, we performed similar experiments in which a fluorometric assay (Jefferson, 1987) was used to measure GUS activity in soluble extracts. Results from one such experiment are summarized in Table 1. Average *GUS* expression in all Km^r transformants was 12-fold higher for the double SAR construct than for the same gene lacking SARs. In addition, the coefficient of variation is reduced by a factor of almost two. Thus,

Table 1. Statistical Comparison of *GUS* Expression and Gene Copy Number for Lines Transformed with the Double SAR or Control Plasmids

Statistic	Control	Double SAR
Sample size	20	29
Mean expression[a]	8.5 ± 6.3	100 ± 33
Standard deviation	28	177
Coefficient of variation[b]	3.3	1.8
Mean gene copy number[c]	70 ± 18	36 ± 6
Standard deviation	82	30
Coefficient of variation	1.2	0.8

[a] Samples were analyzed for GUS activity (± SE) by fluorometric assay. The NT-1 cell line was cotransformed, and 5-mL suspension cultures were started. Cultures were transferred weekly and harvested 56 days after transformation, as described in Methods.

[b] Coefficient of variation = standard deviation/mean (Sokal and Rohlf, 1969).

[c] Samples were analyzed for *GUS* gene copy number by PCR assay, as described in Methods.

we concluded that SARs can reduce the variability inherent in this type of transformation assay while substantially increasing the average level of expression per transformed cell.

Copy Number and Integration Pattern

To determine if increased expression observed with SAR constructs might simply reflect an increase in the number of integrated gene copies, we analyzed genomic DNA from each cell line with a quantitative polymerase chain reaction (PCR) procedure, as described in Methods, using calibration curves similar to those in Figure 3. Representative samples were also subjected to DNA gel blot analysis. Table 1 and Figure 4 show that *GUS* gene copy numbers were actually lower for transformants containing the SAR constructs than they were in the control population. For control transformants, *GUS* copy numbers were distributed over a broad range averaging approximately 70 copies per 1C chromosome complement, while the corresponding values for SAR transformants were more narrowly distributed around a mean value of 36 copies. Combining the 12-fold difference in average expression with this twofold difference in gene copy number (Table 1) gives an overall 24-fold increase in average *GUS* expression per gene copy that can be attributed to the presence of flanking SARs in these constructs.

DNA gel blot analysis confirmed the copy number estimates made by quantitative PCR, but revealed no major differences in integration patterns between the two types of constructs. Figure 5 shows that integration patterns were frequently complex, as has often been reported for direct transformation procedures (Klein et al., 1988; Gordon-Kamm et al., 1990; Saul and Potrykus, 1990; Tomes et al., 1990; Christou, 1992; Klein et al., 1992). In the EcoRI digests shown in Figure 5, bands corresponding in size to intact monomers of the introduced plasmid (indicated by arrows in the figure) most likely arise from sites in which several such units are integrated in tandem. Many of the other bands presumably reflect chimeric fragments resulting from integration of the transforming DNA at various positions in the plant genome. Such bands may or may not contain intact gene copies, so it is difficult to make precise estimates of the number of intact genes in most cell lines. However, there was no obvious difference in the complexity of the patterns obtained in the presence or absence of SARs. Thus, there is no reason to believe that the fraction of integrated DNA comprised of intact genes should differ between SAR and control constructs.

Several lines of evidence are consistent with the interpretation that the *GUS* gene is stably integrated into tobacco genomic DNA. To survive extrachromosomally, the introduced plasmid would have to replicate quite efficiently, because our cell lines were grown for 8 weeks before expression was assayed. ARS-1 is unlikely to serve as an efficient origin of extrachromosomal replication in plant cells, because it does not do so in other heterologous systems (Krysan et al., 1989). In addition, DNA gel blot analysis of nuclear DNA preparations showed no evidence of free plasmid. Identical restriction profiles were obtained from selected cell lines at different times after transformation, and undigested genomic DNA hybridized only to fragments of high molecular weight.

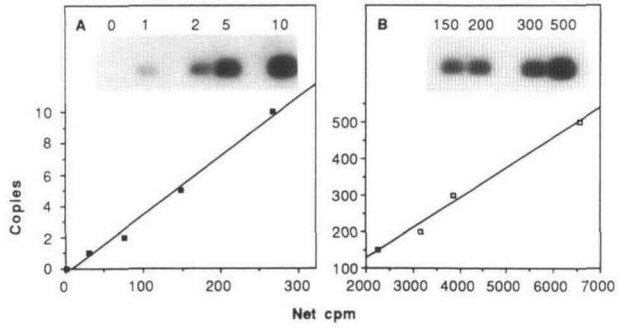

Figure 3. Copy Number Estimation by PCR.

Examples of standard curves used for estimating 35S::*GUS* copy number are shown. Reconstruction standards were prepared by serially diluting the double SAR plasmid into wild-type NT-1 genomic DNA to produce mixtures containing between 1 and 500 copies per 1C chromosome complement of tobacco DNA. Each DNA preparation was amplified and analyzed by gel blot hybridization, as described in Methods. Signal intensities were quantitated with an Ambis radioanalytic scanner.

(A) Standard curve for copy numbers between 0 and 10.

(B) Standard curve for copy numbers between 150 and 500.

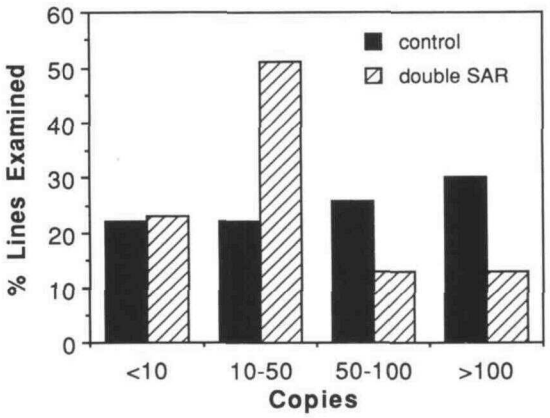

Figure 4. Gene Copy Number Distributions.

Percentage of cell lines with copy numbers in the indicated ranges is plotted against the 35S::*GUS* copy number estimated by the PCR procedure described in Methods and Figure 3. Double SAR lines are represented by hatched bars and control lines by solid bars.

Expression and Gene Copy Number

Studies with animal systems have shown that including SARs in constructs similar to those used in our experiments creates a situation in which expression levels closely reflect the number of gene copies introduced. Such observations are usually interpreted as indicating that SARs insulate the introduced DNA from chromosomal position effects, so that the expression level per gene copy is the same in independent transformants. To determine whether position effects had been eliminated in our cell lines, we measured GUS activities and *GUS* gene copy numbers for cell lines containing either the control or the double SAR construct. These results are plotted as a scatter diagram in Figure 6. It is immediately apparent that neither set of transformants showed expression proportional to copy number. Only two control cell lines had more than 5 units of GUS activity. These lines were estimated to contain three and 17 copies of the gene, whereas many of the remaining control transformants contained much higher copy numbers. Interestingly, the double SAR construct also produced relatively low activities in the five cell lines with more than 50 copies of the gene. It may be significant that three of these lines still showed more GUS activity than control lines with similarly high gene copy numbers. However, by far the largest SAR effect was obtained at copy numbers less than 50, and there was a striking tendency for the most active SAR lines to be tightly clustered in a "window" between approximately 20 and 50 copies.

Transient Expression

We have used transient expression assays to distinguish between conventional enhancer activity and an activity requiring chromosomal integration. Because most transcription in such assays is thought to occur prior to integration, they are thought to reflect chromatin-level events rather poorly, if at all. Figure 7A shows that including one or more SARs does increase *GUS* expression 20 hr after electroporation. However, the effect of a double SAR averages only approximately twofold as compared to the ~12-fold effect observed in stable transformation. It is also noteworthy that the double SAR construct fails to increase expression beyond the level mediated by the 3′ SAR alone; this result contrasts with the greater effect of the double SAR construct in stable transformation experiments (e.g., Figure 2). Even the small effect of SARs in the transient expression experiment of Figure 7A might require integrated DNA,

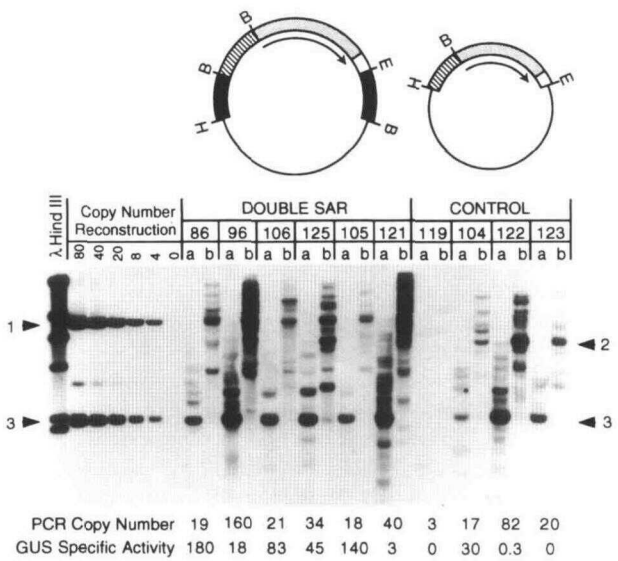

Figure 5. DNA Gel Blot Analysis of Selected Cell Lines.

Cell lines carrying control or double SAR constructs were selected to include a wide range of gene copy numbers and expression levels, as shown below the gel. High molecular weight nuclear DNA was prepared and analyzed by gel blot hybridization with a *GUS* probe, as described in Methods. Maps of the transforming plasmids, with relevant restriction enzyme sites (H, HindIII; B, BamHI; E, EcoRI), are shown above the appropriate lanes. Narrow lines indicate vector sequences; black boxes, SAR (ARS-1) elements; hatched boxes, CaMV 35S promoter; grey boxes, *GUS* sequences; and open boxes, the *nos* terminator. DNA from each cell line was singly digested with EcoRI (lanes b) or triply digested with EcoRI, HindIII, and BamHI (lanes a). Because EcoRI cuts once in each plasmid, single digests of free plasmid or tandemly integrated copies will produce the bands identified by arrows 1 and 2 for the double SAR and control plasmids, respectively. The triple digest produces a *GUS*-hybridizing band identified by arrows numbered 3 from either plasmid. Copy number reconstruction lanes shown on the left contain 5 μg of NT-1 genomic DNA together with appropriate amounts of an equimolar mixture of singly and triply digested double SAR plasmid.

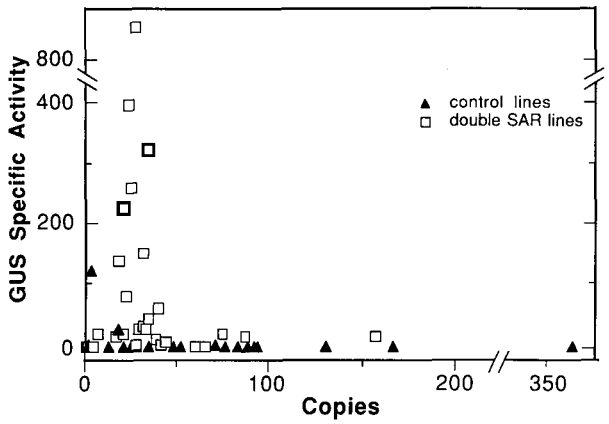

Figure 6. *GUS* Expression as a Function of Gene Copy Number in Stably Transformed Cell Lines.

GUS activity was determined by fluorometry, and gene copy number was determined by the PCR procedure for each transgenic cell line ~8 weeks after transformation, as described in Methods. Double SAR transformants are represented by open squares and control lines by closed triangles.

because some integration might be expected to occur during the course of such an experiment. Consistent with this notion, Figure 7B shows that the difference between SAR and control constructs is small at first, becoming obvious only between 9 and 13 hr of incubation.

Figure 7C compares data from transient expression and stable transformation experiments. The results for stable transformation are taken from Table 1 and are average values for the entire population of cell lines, including those in which no expression was detected. Although *GUS* expression from the control construct is somewhat higher in stably transformed lines than in transient assays, the most dramatic difference is obtained with the double SAR construct. Clearly, the activity of this construct is differentially increased in stably transformed cell populations.

DISCUSSION

GUS gene expression in stably transformed cell lines averaged more than 20-fold higher on a gene copy basis when transformation was performed with the double SAR construct. Significantly, transient expression assays with the same constructs showed only approximately a twofold SAR effect. Thus, the major effect appears to require chromosomal integration. In addition, as shown in Figure 2, we observe considerably larger effects when SAR elements are present on both sides of the reporter gene than when only a single SAR is present. For these reasons and others discussed below, we believe classic enhancer activity cannot account for our data, and that the ARS-1 element most likely exerts an effect on some aspect of chromosome structure and function.

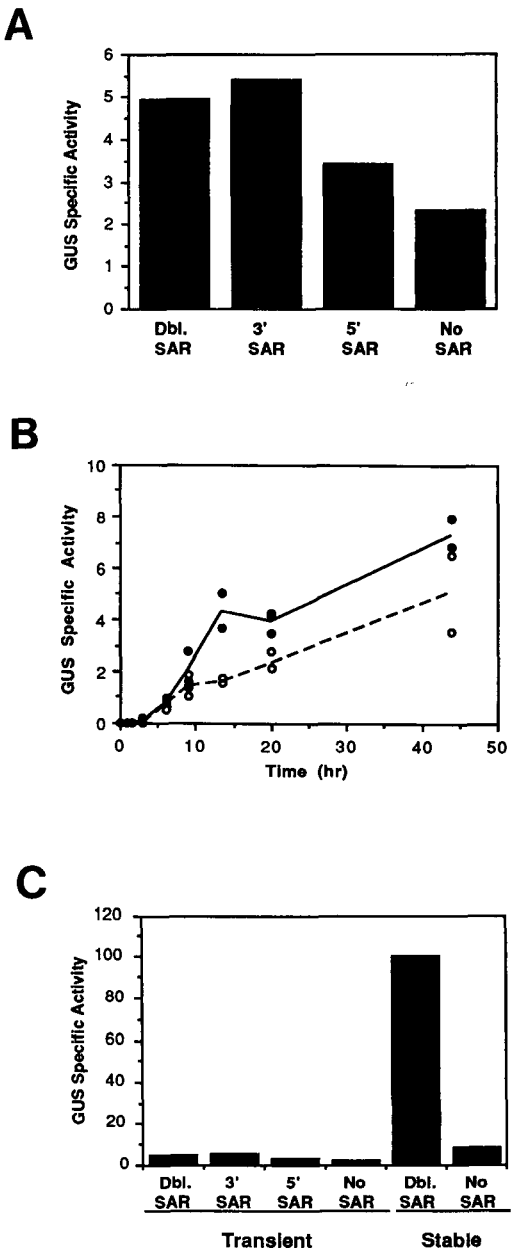

Figure 7. Comparison of Transient and Stable Transformation.

The indicated constructs were introduced into NT-1 protoplasts by electroporation, as described in Methods, and GUS activity was measured by fluorometric assay.

(A) Average *GUS* expression from three experiments for double (Dbl.) SAR, 3′ SAR, 5′ SAR, and control (No SAR) plasmids measured 20 hr after electroporation. Standard error of the mean was less than 15% in each case.

(B) Time course of *GUS* expression after electroporation of double SAR (closed symbols) and control (open symbols) plasmids. Individual data points are from independent experiments.

(C) Comparison of transient and stable expression. Mean GUS activities 20 hr after electroporation are compared to mean values for stably transformed cell lines taken from Table 1.

Our results differ dramatically from those of Breyne et al. (1992) in that we observed a large increase, as opposed to a decrease, in the average expression of reporter gene constructs flanked by SARs. However, the two sets of experiments differ in several respects, which may be critical to an eventual understanding of the differences in the results. Breyne et al. (1992) used an Agrobacterium transformation vector carrying a reporter gene with or without SARs. A large fraction of transformation events selected in this way involve integration events close to active genes (Herman et al., 1990; Kertbundit et al., 1991), which may reflect a tendency for Agrobacterium-based vectors to integrate into active chromatin. In contrast, we used direct DNA transformation and introduced selectable and assayable markers on separate plasmids. This procedure avoids possible effects of T-DNA border sequences and may reduce the linkage between the two markers. However, it results in patterns of integration that are quite different from those obtained with T-DNA vectors.

In particular, it may be significant that most of our transformants contained multiple copies of the introduced genes. Direct transformation with purified DNA is known to produce complex events in which multiple copies of the introduced DNA become integrated at one or a few loci in the recipient genome (Christou and Swain, 1990; Saul and Potrykus, 1990). SAR constructs should thus produce large transgenic loci with many closely spaced SARs, in contrast to the relatively small T-DNA loci that each contain a single pair of SARs. It is easy to imagine that such different structures might have different effects on gene expression.

Another difference between our experiments and those of Breyne et al. (1992) is the source of the SAR; they found their effect with a soybean DNA fragment that showed relatively strong in vitro binding to tobacco nuclear scaffolds. This fragment, although clearly a SAR, may have different overall properties than the ARS-1 element we used in our experiments. As further discussed below, many SAR-containing fragments are multifunctional, and it is not always possible to attribute a given biological effect to scaffold binding per se.

Relation to Cosuppression

SAR constructs gave increased expression only in transformants with copy numbers in a relatively narrow window between approximately 20 and 50 copies per 1C chromosome set (Figure 6). Above this window, *GUS* expression was much reduced, and the activity of the SAR transformants differed only slightly from that of the controls. We interpret the data as indicating a positive dosage effect in SAR transformants up to approximately 30 to 40 copies of the 35S::*GUS* construct, followed by a suppression of activity at higher copy numbers. In the absence of SARs, expression decreased more rapidly, and only lines with very low copy numbers showed strong expression. Results similar to our control data have been reported previously by Linn et al. (1990), who showed that consistently high expression is obtained more frequently in transgenic petunia plants containing a single introduced gene than in similar plants containing multiple copies of the same gene.

It seems likely that the decline in activity at higher copy numbers is an example of the phenomenon of cosuppression, which can be broadly defined as interactions between nonallelic gene copies leading to reversible epigenetic inactivation (Matzke et al., 1989; Jorgensen, 1990; Matzke and Matzke, 1990; van der Krol et al., 1990; Mittelsten Scheid et al., 1991). Although often observed in studies of plant transformation, cosuppression may also account for several epigenetic phenomena involving endogenous genes, such as paramutation and epimutation (Jorgensen, 1990). Premeiotic inactivation and repeat-induced mutation in fungi exhibit similarities to cosuppression (Selker, 1990); however, there are no reports of phenomena resembling cosuppression in animals (Jorgensen, 1990). The absence of cosuppression effects in animal cells may account for several reports documenting a linear relationship between gene copy number and expression of SAR-containing gene constructs (Stief et al., 1989; Phi-Van et al., 1990; Klehr et al., 1991; McKnight et al., 1992). In our experiments, this relationship cannot be extended to high copy numbers, because gene activity was effectively suppressed in these cell lines. However, SAR constructs were apparently less sensitive to cosuppression than control constructs and showed high levels of activity with an overall positive dose effect in cell lines containing up to approximately 30 to 40 copies of the introduced gene.

ARS and Enhancer Models

Many SARs are closely associated with transcriptional enhancers and/or sequences that act as origins of replication in yeast (Gasser and Laemmli, 1986; Amati and Gasser, 1988; Stief et al., 1989; Amati et al., 1990; Brun et al., 1990; Phi-Van et al., 1990; Klehr et al., 1991; McKnight et al., 1992). The ARS-1 element is typical in this respect. Although it contains a well-known yeast SAR (Amati and Gasser, 1988) and exhibits specific binding to tobacco nuclear scaffolds (Hall et al., 1991), it was first defined by its ability to support autonomous replication in yeast (Stinchcomb et al., 1979) and is known to contain binding sites for an abundant nuclear protein with functions both in DNA replication and in transcription of a large number of yeast genes (Rhode et al., 1992). Thus, we must consider the possibility that activities other than scaffold attachment may contribute to ARS-1 effects in plant cells.

Transcriptional enhancer activity seems unlikely to account for the effects we observed. It is conceivable, albeit unlikely, that the ARS element could act as an enhancer strong enough to stimulate gene expression beyond the level already mediated by the 35S promoter. However, such an activity should also manifest itself in a transient expression assay. Because we observed less than twofold stimulation of expression in transient assays, the 12-fold effect we observed in stable transformants (24-fold on a gene copy basis) is not readily attributable to an enhancer effect.

It is more difficult to exclude the possibility that our data reflect an influence of the yeast element on DNA replication. ARS activity is known to be associated with a significant subclass of SARs, perhaps because both scaffold binding and ARS activity require AT-rich regions that confer a distinct helical structure and are particularly susceptible to local denaturation or unwinding (Amati and Gasser, 1988; Amati et al., 1990; Brun et al., 1990; Bode et al., 1992). We do not believe our SAR constructs are maintained as extrachromosomal replicons because they comigrate with high molecular weight genomic DNA in agarose gels and because there is no precedent for a yeast ARS supporting extrachromosomal replication in heterologous systems. However, it is more difficult to exclude the possibility that ARS or SAR sequences might affect chromosomal DNA replication. Because early replication is closely associated with active gene expression (Goldman, 1988; Fangman and Brewer, 1992), altering the time at which the introduced DNA replicates during S phase might conceivably stimulate gene expression.

Scaffold Attachment Models

The simplest models to explain the SAR effect assume that SARs mediate in vivo binding to the nuclear scaffold. The loop domain model, in which SARs are thought to define the ends of chromatin loops corresponding to independent regulatory domains, has already been described in the Introduction. According to this model, scaffold attachment would insulate the introduced DNA from the influence of factors in the chromatin surrounding the site of integration. Because most of the chromatin is in an inactive conformation at any given time, such influences are expected to be predominantly negative and blocking them should tend to increase gene activity.

Because our multicopy transformants are likely to contain tandemly arrayed copies of the introduced constructs (Saul and Potrykus, 1990), they should also contain a series of closely spaced SARs. According to the loop domain model, some or all of these SARs may associate with the nuclear scaffold in vivo to produce a series of domains insulated from the influence of *cis* elements and structural effects from outside the transgenic locus. This arrangement would also result in small loop domains that bring the *cis* regulatory elements close to the scaffold. Consistent with this idea is the observation that genes in small loop domains seem to be expressed at a higher level than genes in large loop domains (Gasser and Laemmli, 1987). Alternatively or in addition, segments of chromatin with multiple points of scaffold attachment might physically resist compaction into heterochromatin, allowing continued access by polymerases and transcription factors. This model would be especially appealing if chromatin compaction proves to be involved in cosuppression, because it would provide a mechanistic explanation for our observation that SAR-containing constructs are more resistant to cosuppression than control constructs.

METHODS

Plasmid Constructs

Reporter plasmids were derived from pBI221 (Jefferson et al., 1987), which contains a chimeric β-glucuronidase (*GUS*) gene under control of the cauliflower mosaic virus (CaMV) 35S promoter (35S) and a 3′ polyadenylation signal derived from the nopaline synthase (*nos*) gene (Depicker et al., 1982). The scaffold attachment region (SAR) fragment was the ARS-1 element from the yeast plasmid YRP7 (Amati and Gasser, 1988). This fragment has previously been shown to bind yeast (Amati and Gasser, 1988) and plant nuclear scaffolds (Hall et al., 1991). The various SAR-containing plasmids (5′ SAR, 3′ SAR, and double SAR) were obtained by inserting the ARS-1 fragment of YRP7 5′ and 3′ of the 35S::*GUS*::*nos* gene in the multiple cloning site of pBC (Stratagene). Cloning procedures were as described by Sambrook et al. (1989).

The selection plasmid was pUCNK1 (Herrera-Estrella et al., 1988) containing a *nos*::*nptII* gene conferring kanamycin resistance.

Transformation

The *Nicotiana tabacum* cell line NT-1 was obtained from G. An (Washington State University, Pullman). Suspension cultures were grown in a medium containing Murashige and Skoog salts (GIBCO Laboratories, Grand Island, NY) supplemented with 100 mg/L inositol, 1 mg/L thiamine HCl, 180 mg/L KH_2PO_4, 30 g/L sucrose, and 2 mg/L 2,4-dichlorophenoxyacetic acid. The pH was adjusted to 5.7 before autoclaving. Cells were subcultured once per week by adding 3 mL of inoculum to 100 mL of fresh medium in 500-mL Erlenmeyer flasks. The flasks were placed on a rotary shaker at 125 rpm at 27°C and a light intensity of 47 μmol m^{-2} sec^{-1}.

Four-day-old cells, in early-log phase, were transformed by microprojectile bombardment. Aliquots of 50 mL were centrifuged, and the pellet was resuspended in fresh culture medium at a concentration of 0.1 g/mL. Aliquots of 0.5 mL were spread as monolayers onto sterile lens paper that had been placed on culture medium solidified with 2% agar in 60-mm-diameter Petri plates. Plated cells were kept at room temperature for 3 hr prior to bombardment. Microprojectile bombardment was performed with a particle accelerator (PDS-1000; DuPont) using 1500 psi rupture disks with the sample positioned 5.5 cm from the launch assembly.

Each batch of cells was cotransformed with a mixture of "expression" and "selection" plasmids (Figure 1). A plasmid containing the *GUS* gene driven by the CaMV 35S promoter (Benfey and Chua, 1989) was used to measure expression, while a plasmid containing a neomycin phosphotransferase gene (*nptII*) driven by the *nos* promoter (Depicker et al., 1982) was used to select for cells that had stably integrated exogenous DNA. Cotransformation mixtures contained a 4:1 molar ratio of *GUS* reporter plasmid to *nptII* selection plasmid. Thus, each aliquot of a standard double SAR transformation mixture consisted of 68 ng of selection plasmid and 432 ng of double SAR plasmid. Appropriate quantities of each DNA (5 μL) were mixed and precipitated with 50 μL of 2.5 M $CaCl_2$ and 20 μL of 0.1 M spermidine onto 1.0-μm gold microprojectiles. After bombardment, the Petri plates were sealed with parafilm and incubated for 24 hr at 27°C under constant light. Using the lens paper, cells were then transferred to fresh plates containing media supplemented with 300 μg/mL kanamycin. Independent

kanamycin-resistant (Km^r) microcalli began to appear in ~3 weeks, at which time they were transferred to fresh plates containing kanamycin medium. After 1 additional week, suspension cultures were started by inoculating 1 mL of broth supplemented with 100 μg of kanamycin. Growth in liquid medium ensured that established cell lines were uniformly resistant to kanamycin. Once established, the suspension cultures were transferred weekly for 4 additional weeks, using 3% (v/v) inocula in 5 mL of broth supplemented with 100 μg/mL kanamycin.

DNA Isolation

Cell cultures (5 mL) were harvested by filtration 7 days after inoculation and frozen in liquid nitrogen. Frozen cells were broken by grinding under liquid nitrogen with a mortar and pestle. A crude nuclear fraction was then obtained using a nuclei isolation buffer (NIB) similar to that described by Hall et al. (1991) but containing 3% β-mercaptoethanol and lacking protease inhibitors. The sample was suspended in 5 mL of NIB and centrifuged at 500*g* for 5 min at 4°C in a Beckman GPR centrifuge.

The pellet of crude nuclei was resuspended in 1 mL of NIB and then gently mixed with an equal volume of lysis buffer containing 0.2 M Tris, pH 8.0, 50 mM sodium-EDTA, 1 M NaCl, and 2% sarkosyl. After lysis, DNase-free RNase was added to a final concentration of 10 μg/mL, and the samples were incubated for 1 hr at 37°C prior to the addition of proteinase K to 500 μg/mL and incubation for a further 2 hr. Each sample was then extracted sequentially with equal volumes of phenol, phenol/chloroform/isoamyl alcohol (25:24:1), and chloroform/isoamyl alcohol (24:1). DNA was precipitated by adding 0.1 volume of 3 M sodium acetate, pH 5.2, followed by 1 volume of isopropanol. The fibrous precipitate was recovered on a glass rod, dissolved in 250 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), and dialyzed overnight at 4°C against two changes of the same buffer.

Gene Copy Number Analysis

Estimates of *GUS* gene copy number were obtained for all cell lines by a quantitative polymerase chain reaction (PCR) procedure and confirmed for representative lines by genomic DNA gel blot analysis. The PCR procedure used primers located in the 35S promoter (5′-TCAAGATGCCTCTGCCGACA-3′) and in the translated region of the *GUS* gene (5′-TCACGGGTTGGGGTTTCTAC-3′), as shown in Figure 1. Each reaction mixture (100 μL) contained 0.2 mM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 0.1 mM of each oligonucleotide primer, 2.5 units of *Taq* polymerase, 50 mM KCl, 10 mM Tris-HCl, pH 8.8, and 100 ng genomic DNA. Each cycle consisted of 2 min at 94°C, 2.5 min at 50°C, and 3 min at 72°C. Reactions were terminated following a final extension step of 7 min at 72°C.

PCR was limited to 15 cycles to avoid substrate exhaustion, and amplification products were visualized by blotting and hybridization with a ^{32}P-labeled DNA probe. Reconstruction standards were prepared by serially diluting DNA from the double SAR plasmid into wild-type NT-1 genomic DNA to introduce between 1 and 500 *GUS* genes per 1C chromosome complement (5 pg) of tobacco DNA (Arumuganathan and Earle, 1991). PCR reactions were done simultaneously for standards and unknowns. Data from a preliminary set of reactions were used to sort the DNA samples into groups with similar copy numbers, and a second set of reactions was then performed with more closely spaced standards. Grouped samples and standards from these reactions were run on the same gel and analyzed as described above. Hybridization signals were quantitated on a radioanalytical scanner (Ambis, San Diego, CA), and final copy number estimates were calculated using linear regression analysis. Examples are shown in Figure 3.

DNA gel blot analysis was performed using a digoxigenin nonradioactive detection system (Genius Nonradioactive DNA Labeling and Detection Kit, Boehringer Mannheim). Genomic DNA from individual transformants was digested with appropriate restriction enzymes, purified by phenol/chloroform extraction, precipitated with ethanol, and redigested with the same restriction enzymes. Five-microgram aliquots were electrophoresed on 0.75% agarose gels. The blots were transferred by capillary action and cross-linked with a Stratalinker (Stratagene). Prehybridization and hybridization were done at 65°C in a buffer containing 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 1% blocker (Boehringer Mannheim), 0.1% sarkosyl, and 0.02% SDS. Digoxigenin-labeled probes were prepared by the random primer technique according to the Boehringer Mannheim protocol. The template was a PCR-amplified *GUS* coding sequence. Blots were washed twice for 15 min at room temperature in 2 × SSC, 0.1% SDS, and twice at 65°C in 0.15 × SSC, 0.1% SDS. Bands were detected by an immunochemiluminescent method according to the supplier's protocol.

GUS Assays

For fluorometric analysis, frozen cells were ground in liquid nitrogen as described for DNA extraction. Approximately 50 mg of the resulting powder was resuspended in 600 μL of GUS extraction buffer containing 50 mM $NaPO_4$, pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine (w/v), and 0.1% Triton X-100 (w/v) and sonicated twice for 10 sec. The extract was clarified by treatment with insoluble polyvinylpolypyrrolidone and centrifuged. GUS activity was determined with the fluorometric assay described by Jefferson (1987) using methylumbelliferone glucuronide as substrate.

For histochemical analysis, samples of callus were incubated for 24 hr at 37°C in sterile microcentrifuge tubes containing 200 μL of a 0.5-mg/mL solution of 5-bromo-3-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) (Jefferson, 1987).

Transient Expression

Protoplasts for electroporation were prepared from 4-day-old NT-1 suspension cultures by a procedure similar to that of Hall et al.(1991). Cells from 100 mL of culture were harvested by centrifugation at 300*g* for 2 min in a Beckman GPR centrifuge equipped with a GH3.7 rotor, washed twice in 100 mL of 0.4 M mannitol, and resuspended in an equal volume of protoplasting solution containing 0.4 M mannitol, 20 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.5, 1% cellulase (Onozuka RS, Kanematsu-Gosho, Los Angeles, CA), and 0.1% pectolyase Y23 (Onozuka). They were then incubated at 27°C for 30 to 60 min with shaking at 150 rpm. The resulting protoplasts were washed twice in protoplast buffer containing 0.4 M mannitol by centrifuging at 300*g* for 5 min. The final sample was diluted by adding an equal volume of 2× electroporation buffer (273 mM NaCl, 5.36 mM KCl, 2.94 mM KH_2PO_4, 15.5 mM Na_2HPO_4, 0.4 M mannitol, pH 6.5) to a final concentration of 2 × 10^6 cells per mL.

Each electroporation used 80 μg of sheared *Escherichia coli* carrier DNA and ~20 μg of the plasmid DNA mixture to be tested. One milliliter of protoplasts was added to the cuvette and mixed with

100 μL of DNA in TE buffer; the mixture was then left on ice for 5 min. Electroporation was done in a Cell-Porator (Bethesda Research Laboratories) at 250 V and 1180 μF. Cuvettes were placed on ice for 15 min immediately after treatment. Aliquots (400 μL) of electroporated protoplasts were then transferred to 60-mm-diameter Petri plates containing 4 mL of culture medium with 0.4 M mannitol. After incubation for various time periods, protoplasts were collected by centrifugation at 300*g* for 5 min at 4°C. Each pellet was suspended in 600 μL of GUS extraction buffer, and GUS activity was assayed by the fluorometric procedure described above.

ACKNOWLEDGMENTS

We thank Mark Dickson for helping with the gene gun experiments; Dolores Sowinski for helping with the photographs; Susan Gasser, Bruno Amati, and Wayne Fitzmaurice for supplying plasmids; and Marie Petracek and Lynn Dickey for helpful suggestions on our manuscript. This work was supported by a research and training grant from the McKnight Foundation and by funds from U.S. Department of Agriculture Competitive Research Grants No. 91-37301-6377 and No. 92-37301-7710.

Received February 24, 1993; accepted April 14, 1993.

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